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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)

Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Wa5 ENCODED BY NOVEL ENU-INDUCED ANTIMORPHIC ALLELE OF THE EPIDERMAL GROWTH FACTOR RECEPTOR WITH SPECIFICITY FOR HOMODIMERIC INTERACTIONS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

[X] Specification *Number of Pages* 53
[ ] Drawing(s) *Number of Sheets* _____
[ ] Application Data Sheet. See 37 CFR 1.76
[ ] CD(s), Number _____
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[X] Applicant claims small entity status. See 37 CFR 1.27.
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Respectfully submitted,

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Date 02/17/04

REGISTRATION NO. 39,395 *(If appropriate)*

Docket Number: 421/99 PROV

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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Patty Wilson

Mail Stop Provisional Application
Commissioner for Patents
P.O. Box 1450
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Re: U.S. Provisional Patent Application for
Wa5 ENCODED BY A NOVEL ENU-INDUCED ANTIMORPHIC ALLELE OF THE EPIDERMAL GROWTH FACTOR RECEPTOR WITH SPECIFICITY FOR HOMODIMERIC INTERACTIONS
Our Ref. No. 421/99 PROV

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Please find enclosed the following:

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Respectfully submitted,

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Patty Wilson

Statements of Invention

1. A method for modulating an activity of an epidermal growth factor receptor (EGFR) polypeptide, the method comprising contacting the EGFR polypeptide with a *Egfr*^{wa5} polypeptide, wherein the *Egfr*^{wa5} polypeptide comprises a D833G amino acid change or a change in an analogous amino acid residue, whereby an activity of the EGFR polypeptide is modulated.

2. The method of Statement 1, wherein the EGFR polypeptide is present within a cell.

3. The method of Statement 2, wherein the cell overexpresses EGFR.

4. The method of Statement 2, wherein the cell is selected from the group consisting of a tumor cell, a neoplastic cell, and a pre-neoplastic cell.

5. A method of inhibiting the growth and/or proliferation of a cell, the method comprising contacting the cell with a *Egfr*^{wa5} polypeptide, wherein the *Egfr*^{wa5} polypeptide comprises a D833G amino acid change or a change in an analogous amino acid residue.

6. The method of Statement 5, wherein the cell is selected from the group consisting of a tumor cell, a neoplastic cell, and a pre-neoplastic cell.

7. The method of Statement 5, wherein the cell is an intestinal tumor cell.

8. The method of Statement 5, wherein the cell is present in a subject.

Title: **Wa5 is encoded by a novel ENU-induced antimorphic allele of the epidermal growth factor receptor with specificity for homodimeric interactions**

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Running Title: Waved-5 allele

Key Words: *Egfr*, antimorph, dominant negative, *Apc*^{Min}, *Erbb2*, waved

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ABSTRACT

Mice heterozygous for the N-ethyl-N-nitrosourea-induced *Waved-5* (*Wa5*) mutation, isolated in a screen for dominant, visible mutations, exhibit a wavy coat similar to mice homozygous for the recessive $Tgfa^{wa1}$ or $Egfr^{wa2}$ alleles. In this study, we show that *Wa5* is a new allele of *Egfr* containing a mis-sense mutation within a highly conserved DFG motif coding for the tyrosine kinase domain. *In vivo* analysis demonstrates that $Egfr^{Wa5}$ functions as an antimorphic allele, recapitulating many abnormalities associated with reduced EGFR activity. Consequently, $Egfr^{Wa5}$ enhances $Egfr^{wa2}$ compound or $Tgfa^{tm1Dcl}$ double mutants exposing additional EGFR-dependent phenotypes. Using *in vitro* biochemical analysis, we show that $Egfr^{Wa5}$ produces a kinase dead, dominant negative receptor. The dominant negative activity of Wa5 is specific for EGFR homodimers as Wa5 does not inhibit ERBB2 heterodimers. These data provide genetic support for a model of EGFR activation based upon tetrameric or oligomeric complexes. Thus, the $Egfr^{Wa5}$ allele is a novel and potent homodimer-specific antimorphic allele that provides new insights into macro-molecular structural assemblies required for EGFR activation.

Introduction

The epidermal growth factor receptor (EGFR) is the prototypical type-I receptor tyrosine kinase and the first member of the ERBB family of receptors to be identified (Coussens et al. 1985; Kraus et al. 1989; Carpenter and Wahl 1990; Plowman et al. 1990; Plowman et al. 1993; Gullick 1998). EGFR, like other ERBB receptors, is required for normal mammalian development as demonstrated using targeted alleles such as *Egfr*^{tm1Mag} (Threadgill et al. 1995). Our analysis of EGFR-deficient mice revealed that the genetic background strongly influences the timing of lethality, ranging from peri-implantation to early post-natal periods. The major developmental abnormality associated with EGFR deficiency is abnormal placental development that causes mid-gestation lethality on many genetic backgrounds (Strunk et al. submitted).

Similarly, the recessive hypomorphic allele *Egfr*^{wa2}, that arose spontaneously, has been used to identify important functions for EGFR in protection from and repair of tissue damage (Helmrath et al. 1997; Egger et al. 2000; Wang et al. 2003). However, the mutant receptor encoded by *Egfr*^{wa2} has almost normal *in vivo* activity as determined by measuring ligand-induced kinase activity (Luetteke et al. 1994; Fowler et al. 1995; Keegan et al. 2000). The activity of the wa2 receptor has been reported to be as high as 90% of wild-type EGFR and consequently, mice heterozygous for either *Egfr*^{wa2} or the *Egfr*^{tm1Mag} null allele have no abnormalities. Since the wa2 receptor appears to have only minor impairments in kinase activity, it has provided limited insights into the structural requirements and mechanisms responsible for normal kinase activation.

Although significant insights into gene function are gained from studying targeted alleles, the full range of function for gene products are better investigated using allelic series. The classical and proven method for generating allelic series is through random mutagenesis and in the mouse, N-ethyl-N-nitrosourea (ENU) is the favored mutagen (Russell and Montgomery 1982). ENU has been used in mice to generate allelic series by

sequence-based screens for mutations in specific genes using mutagenized embryonic stem cells (Chen et al. 2000b; Munroe et al. 2000) and by phenotype-based screens using mutagenized mice (Marker et al. 1997; Hallsson et al. 2000; Rajaraman et al. 2002). We previously characterized a new allele of *Egfr*, $Egfr^{Dsk5}$, recovered in a large-scale screen for dominant visible mutations (Fitch et al. 2003). $Egfr^{Dsk5}$ heterozygous and homozygous mice are relatively normal except for a wavy coat and altered footpad pigmentation. Molecularly, we showed that this allele has a hyper-activatable kinase when assayed *in vitro*. However, *in vivo*, the $Egfr^{Dsk5}$ allele is dramatically down-regulated through negative feedback regulation while ligand-induced receptor activity is elevated. These studies into the regulation of the $Egfr^{Dsk5}$ allele provided novel insights into the regulation of EGFR based upon total kinase activity.

The mechanism by which EGFR becomes activated is currently believed to be via pre-formed inactive dimers that undergo an extracellular conformational change upon ligand binding (Martin-Fernandez et al. 2002; Yu et al. 2002). The change in extracellular conformation causes a concomitant cytoplasmic conformational change, transmitted via the transmembrane domain, that activates the intrinsic tyrosine kinase. The activated dimers then trans-phosphorylate their respective dimerization partners. However, alternative mechanisms have been proposed based upon structural modeling and biochemical data suggesting that EGFR activation may involve formation of tetramers or higher-order complexes (Honegger et al. 1989; Murali et al. 1996; Huang et al. 1998).

In the current study, we have identified a second dominant allele of *Egfr*, termed $Egfr^{Wa5}$, that was recovered from a mutagenesis screen for dominant, visible mutations (Thaung et al. 2002), independent of the screen that produced $Egfr^{Dsk5}$ (Fitch et al. 2003). This allele was detected because the eyes were open at birth and by the existence of a wavy coat, similar to the phenotypes caused by $Egfr^{wa2}$, $Tgfa^{wa1}$ and $Egfr^{Dsk5}$ alleles. Sequence analysis showed that $Egfr^{Wa5}$carries a novel mutation altering the highly-conserved DFG motif within the activation loop of the EGFR kinase domain. We demonstrate that Wa5

lacks kinase activity and that homozygous $Egfr^{Wa5}$ embryos die during gestation with phenotypes indistinguishable from embryos homozygous for the targeted null allele $Egfr^{tm1Mag}$ (Threadgill et al. 1995). However, unlike previous alleles, we establish that $Egfr^{Wa5}$ encodes a novel antimorphic allele of *Egfr* that is specific to homo-dimeric complexes. Thus, analysis of Wa5 identifies DFG as a motif critical for the formation of active receptor complexes and provides support for a model of EGFR activation requiring tetrameric or high-order complexes for efficient kinase activation. Based upon genetic and molecular data for $Egfr^{Wa5}$, taken together with recent data implicating the existence of inactive pre-dimers, we propose a new model for EGFR activation whereby pre-dimers coalesce into tetramers or high-order complexes upon ligand binding and that these complexes activate the intrinsic kinase of EGFR.

Results

Wa5 *has a point mutation in a highly conserved residue within* Egfr *and causes embryonic and peri-natal lethality*

Previous analysis of ENU-induced mutations causing dominant visible phenotypes detected *Wa5* as a mutation causing open eyes at birth, curly whiskers, and a wavy coat similar to the recessive $Tgfa^{wa1}$ and $Egfr^{wa2}$ mutations (Thaung et al. 2002). Since genetic mapping localized the mutation to proximal Chromosome 11 near the *Egfr* locus, we performed a complementation cross with an $Egfr^{tm1Mag}$ null allele (Table 1). The *Wa5* allele failed to complement $Egfr^{tm1Mag}$, supporting that *Wa5* is a new allele of *Egfr* ($Egfr^{Wa5}$). We subsequently sequenced the entire coding region from $Egfr^{Wa5}$ transcripts and identified a single point mutation that creates a mis-sense mutation (GAT to GGT) in exon 21; this change results in a D833G amino acid change within the tyrosine kinase domain (Fig. 1A), altering a DFG motif that is ubiquitously conserved in all functional protein kinases (Hanks

et al. 1988). The Lys^{723} residue, commonly mutated to generate kinase dead receptors for *in vitro* studies (Honegger et al. 1987; Cheng and Koland 1998), binds Mn·ATP that is aligned within the active site by Asp^{833} for catalysis at Asp^{815} (Coker et al. 1994). The previously characterized $Egfr^{wa2}$ and $Egfr^{Dsk5}$ mutations, creating changes at Val^{743} and Leu^{839}, probably alter the structure of the active site since they are in an alpha-helix (αc) and the activation loop, respectively, rather than the catalytic loop (Fig. 1B).

Tyrosine kinases lacking the conserved DFG motif are kinase dead (Kroiher et al. 2001) and hypothesized to negatively regulate active kinases when oligomerized. In this manner, $Egfr^{Wa5}$ may be acting as an antimorphic allele, distinctly different than the previously characterized dominant hypermorphic mutation, $Egfr^{Dsk5}$ (Fitch et al. 2003).

Since $Egfr^{Wa5}$ failed to complement the $Egfr^{tm1Mag}$ null allele, we characterized the survival of $Egfr^{Wa5}$ homozygous embryos generated from $Egfr^{Wa5}$/+ intercrosses. Morphologically normal $Egfr^{Wa5/Wa5}$ embryos were observed at 10.5 dpc in expected Mendelian ratios (Table 2). Lethality of the majority of $Egfr^{Wa5/Wa5}$ embryos (71%) occurs between 10.5 and 12.5 dpc; a similar age of embryonic lethality was found to be caused by homozygosity for the $Egfr^{tm1Mag}$ null allele (Threadgill et al. 1995). Morphologically abnormal and severely degenerated embryos at 10.5 to 14.5 dpc were either $Egfr^{Wa5}$/+ (25%) or $Egfr^{Wa5/Wa5}$ (75%). Although a few $Egfr^{Wa5/Wa5}$ embryos developed to later stages of gestation, no $Egfr^{Wa5/Wa5}$ embryos survived post-natally; despite being grossly normal in size, three newborn $Egfr^{Wa5/Wa5}$ pups were found dead shortly after birth. This result is consistent with that observed for the $Egfr^{tm1Mag}$ null allele where embryos homozygous for $Egfr^{tm1Mag}$ display variable embryonic and early post-natal lethality that is dependent upon genetic background (Strunk et al. submitted).

$Egfr^{Wa5}$ *heterozygous and homozygous embryos have abnormal placental architecture*

Since the timing of lethality of *Egfr*$^{Wa5/Wa5}$ embryos matched that of *Egfr*$^{tm1Mag/tm1Mag}$ embryos succumbing to placental defects, we analyzed the morphology of placentae from *Egfr*^{Wa5} mutants at 12.5 dpc by hematoxylin and eosin staining. The placentae from phenotypically normal (Fig. 2B, C) and degenerating (Fig. 2D) *Egfr*^{Wa5} embryos were examined. Wild-type *Egfr* placentae showed normal development of the four major placental layers: decidua, trophoblast giant cells, spongiotrophoblast and labyrinthine layers at 12.5 dpc (Fig. 2A). However, histological analysis of both *Egfr*^{Wa5}/+ and *Egfr*$^{Wa5/Wa5}$ placentae showed a reduction in the size of the spongiotrophoblast layer and a variably compromised labyrinthine layer. Trophoblast giant cells of normal appearance were present in both the *Egfr*^{Wa5}/+ and *Egfr*$^{Wa5/Wa5}$ placentae (Fig. 2, arrowheads). Within the compromised labyrinthine layer of *Egfr*^{Wa5} mutants, we observed multinucleated labyrinthine trophoblast cells in addition to an overall reduction in placental thickness. Similar to observations in *Egfr*^{tm1Mag} homozygotes, we observed variation in placental architecture across both the *Egfr*^{Wa5}/+ and *Egfr*$^{Wa5/Wa5}$ placentae; phenotypes ranged from mild defects to severely affected with significant histological dysmorphology. Runted *Egfr*$^{Wa5/Wa5}$ embryos had more profound placental defects as compared to *Egfr*$^{Wa5/Wa5}$ littermates of normal size. Placentae from runted embryos were thinner with a significant reduction in the spongiotrophoblast layer and exhibited formation of multiple blood pockets within the labyrinthine layer, suggesting that *Egfr*^{Wa5} embryonic lethality is caused by abnormal placental development. These results are consistent with *Egfr*^{Wa5} acting as a null allele when homozygous. However, unlike placentae from *Egfr*^{tm1Mag}/+ embryos that support normal placental development, placentae from *Egfr*^{Wa5}/+ embryos exhibited mildly abnormal placentae, providing further suggest that *Egfr*^{Wa5} is functioning as an antimorphic allele.

Egfr^{Wa5} *allele enhances the recessive* Egfr^{wa2} *hypomorphic and* Tgfa^{tm1Dcl} *null phenotypes*

On a mixed genetic background, *Egfr*^{wa2} homozygous pups surviving to adulthood are fertile, and only show defects in hair follicle morphogenesis (Luetteke et al. 1994). Therefore, to determine the effects of further reduction in EGFR activity, we crossed *Egfr*^{wa2}/+ females with *Egfr*^{Wa5}/+ males to generate *Egfr*$^{wa2/Wa5}$ compound heterozygotes (Table 3). When compared to wild-type littermates, only half of the predicted *Egfr*$^{wa2/Wa5}$ compound pups were born although those surviving were normal in size at birth (data not shown). Nine of 16 *Egfr*$^{wa2/Wa5}$ pups died during the first two days after birth, while three *Egfr*$^{wa2/Wa5}$ pups became progressively more runted, dying between two and three weeks of age. Four of 16 *Egfr*$^{wa2/Wa5}$ compound mutants, including three males and one female, survived as long as three months after birth. These *Egfr*$^{wa2/Wa5}$ compound mutants showed severe growth retardation with a body weight ranging from 30 to 50% of the control littermates. Although hair development of *Egfr*$^{wa2/Wa5}$ pups was initially similar to *Egfr*$^{wa2/wa2}$ pups, *Egfr*$^{wa2/Wa5}$ compound mutant mice underwent a complete hair loss around eight-weeks of age (Fig. 3A); new hair growth resumed at two months of age. Craniofacial malformations including an underdeveloped lower jaw and narrow, elongated and assymetric snouts were also observed in *Egfr*$^{wa2/Wa5}$ compound mutant mice (Fig. 3B), similar to the phenotype described previously in *Egfr* nullizygous mice (Miettinen et al. 1999). The only female *Egfr*$^{wa2/Wa5}$ mutant surviving post-weaning showed a delay in vaginal opening until 11 weeks of age, an increase in severity of a phenotype reported for *Egfr*$^{wa2/wa2}$ mice that show delayed vaginal opening at 7.5 weeks (Apostolakis et al. 2000). Neither gender produced offspring by three months of age suggesting they may also have fertility defects.

Similar to the *Egfr*^{wa2} mutation, TGFA deficiency gives rise to coat and eye abnormalities (Luetteke et al. 1993; Mann et al. 1993). Since TGFA is one of the major ligands for EGFR, we examined the phenotypic severity of *Tgfa*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ double mutants produced by crossing *Tgfa*^{tm1Dcl} nullizygous females to *Egfr*^{Wa5}/+, *Tgfa*^{tm1Dcl}/+ males. Double mutant *Tgfa*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ pups were born at expected ratios and were

normal in size at birth. However, these mice had reduced post-natal growth rates resulting in a noticeable decrease in body size by one week of age relative to their littermates. Similar to *Egfr*$^{wa2/Wa5}$ compound mutants, *Tgfr*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ double mutants showed an approximate 25% reduction in body weight by six weeks of age when compared to *Tgfa*^{tm1Dcl} homozygous or *Egfr*^{Wa5} heterozygous littermates (Fig. 3C). Additionally, two *Tgfr*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ double mutants showed smaller lower jaws. Interestingly, unlike body weight, the coat phenotype of the double mutant pups was not exacerbated and remained indistinguishable from *Egfr*^{Wa5}/+ *or* *Tgfa*$^{tm1Dcl/tm1Dcl}$ single mutant mice.

Egfr^{Wa5} *allele reduces the multiplicity but not growth of* Apc^{Min}*-meditated intestinal tumors*

We previously showed that *Apc*^{Min}-mediated intestinal tumor multiplicity is dramatically reduced in *Egfr*$^{wa2/wa2}$ mice (Roberts et al. 2001). Therefore, to functionally verify the reduced activity of *Egfr*^{Wa5}, we crossed *Apc*^{Min}/+ with *Egfr*^{Wa5}/+ mice to generate *Apc*^{Min}/+, *Egfr*^{Wa5}/+ double heterozygotes. Three-month-old *Apc*^{Min}/+, *Egfr*^{Wa5}/+ mice had a 46% reduction in the average number of macroscopic intestinal polyps (> 0.3 mm) compared to *Apc*^{Min}/+ littermates wild-type for *Egfr* (*Egfr*^{Wa5}/+, 7.4 ± 1.7; *Egfr*+/+, 13.7 ± 2.6; P = 0.03; Fig. 4A). Since the cross was segregating alleles at the major *Apc*^{Min} modifier locus, *Mom1*, each mouse was genotyped for resistance (r) or susceptibility (s) at the *Mom1* locus. On a *Mom1*$^{r/s}$ heterozygous background, *Apc*^{Min}/+, *Egfr*^{Wa5}/+ mice showed 53% fewer intestinal polyps than *Apc*^{Min}/+ mice, while on a *Mom1*$^{s/s}$ background, *Apc*^{Min}/+, *Egfr*^{Wa5}/+ mice showed a 32% reduction in tumor number when compared to *Apc*^{Min}/+. This result is consistent with our previous observation of a two-fold greater inhibition in polyp number by *Egfr*$^{wa2/wa2}$ in combination with *Mom1*^{r} compared to *Mom1*^{s} (Roberts et al. 2001). *Apc*^{Min}/+ mice on a *Mom1*$^{r/s}$ background showed a 42% reduction in polyp multiplicity, independent of *Egfr*^{Wa5} status, compared to a *Mom1*$^{s/s}$ background (P = 0.03) consistent with a previous

report (Gould et al. 1996). No gender-dependent differences in polyp multiplicity were observed (data not shown).

Like our previous report using *Egfr*^{wa2}, mice carrying one *Egfr*^{Wa5} allele caused no reduction in tumor size; the average polyp diameter in *Apc*^{Min}/+, *Egfr*^{Wa5}/+ mice was 0.97 ± 0.07 mm compared to 1.05 ± 0.04 mm in *Apc*^{Min}/+ mice (P = 0.17; Fig. 4B). This result also supports our previous work suggesting that there is a critical window during tumor establishment when EGFR activity is essential for a sub-set of tumors; tumor polyps that survive past establishment show EGFR-independent growth. *Apc*^{Min}/+ mice on a *Mom1*$^{r/s}$ background showed a 16% reduction in polyp size, independent of *Egfr*^{Wa5} status, compared to a *Mom1*$^{s/s}$ background (P = 0.0003) as previously reported (Gould et al. 1996). There were no significant differences in polyp size related to gender (data not shown).

In vivo *tyrosine kinase activity is attenuated in* Egfr^{Wa5} *heterozygous mutants*

Exposure of exogenous EGF rapidly induces tyrosine phosphorylation of EGFR, along with 55 kD and 120 kD proteins, in multiple tissues of newborn mice (Donaldson and Cohen 1992; Luetteke et al. 1994). Although high doses of EGF induce equivalent EGFR phosphorylation in *Egfr*$^{wa2/wa2}$ and *Egfr* wild-type neonates, low doses of exogenous EGF result in diminished EGFR phosphorylation in *Egfr*$^{wa2/wa2}$ mice, particularly in skin (Luetteke et al. 1994). In order to characterize the *in vivo* phosphorylation levels of the *Egfr*^{Wa5} mutant receptor, we injected wild-type and *Egfr*^{Wa5}/+ littermates subcutaneously with either PBS or EGF at two or seven days of age. Similar to EGFR activity in *Egfr*$^{wa2/wa2}$ mice, phosphorylation levels of EGFR and other proteins in *Egfr*^{Wa5}/+ mice were greatly attenuated at lower doses of EGF (0.5 or 1.0 μg/g body weight) in liver and skin (Fig. 5A). However, at pharmacological doses of EGF (10 μg/g body weight), tyrosine phosphorylation of EGFR and the 55 kD protein was also greatly attenuated in *Egfr*^{Wa5}/+ mice (Fig. 5B), in contrast to the normal level of tyrosine phosphorylation previously

observed in $Egfr^{wa2/wa2}$ mice (Luetteke et al. 1994). Total levels of EGFR were identical between wild-type and $Egfr^{Wa5}$/+ mice indicating that attenuation of tyrosine phophorylation in $Egfr^{Wa5}$/+ is not due to differential receptor expression but to an inhibitory affect of the Wa5 receptor on wild-type EGFR.

Egfr^{Wa5} *codes for a kinase dead, dominant negative receptor*

All *in vivo* analyses suggested that the $Egfr^{Wa5}$ allele reduces total EGFR activity, even when heterozygous with an *Egfr* wild-type allele. In stark contrast, the $Egfr^{tm1Mag}$ null and $Egfr^{wa2}$ hypomorphic alleles have no detectable heterozygous phenotype. Thus, $Egfr^{Wa5}$ satisfies the genetic definition of an antimorphic allele and would be one of only three known mammalian antimorphic alleles; the other two validated examples are the T^c and $Clock^{mlJl}$ alleles (MacMurray and Shin 1988; King et al. 1997). Therefore, to assess the mechanism by which $Egfr^{Wa5}$ inhibits wild-type EGFR signaling, we transiently tranfected wild-type human *EGFR*, $EGFR^{kd}$ and $EGFR^{Wa5}$ expression vectors into CHO cells for *in vitro* analysis. The $EGFR^{kd}$ mutation was used as a control for an inactive kinase as this mutant cannot utilize ATP and thus is kinase dead (Honegger et al. 1987). Since CHO cells lack endogenous detectable EGFR, total EGFR levels proportionally increased in wild-type, kd, and Wa5 EGFR-expressing cells with increasing quantities of their respective expression vector (Fig. 6A), indicating that the mutations in $EGFR^{kd}$ and $EGFR^{Wa5}$ do not impair EGFR translation. Because CHO cells express endogenous EGFR ligands, the phosphorylation of wild-type EGFR increased proportionally depending on the quantity of transfected expression vector in the absence of additional exogenous EGF treatment. In contrast, kd and Wa5 mutants had no detectable phosphorylation, suggesting that they are inactive kinases.

Since receptor dimerization is essential for activation of EGFR kinase activity under normal conditions, we performed cross-linking assays using wild-type, kd and Wa5 EGFR expressing cells to determine whether Wa5 has an inactive kinase due to an inability to form

ligand-dependent dimers. CHO cells were transiently transfected with 0.5 μg of each expression vector and treated 24 hours late with the cross-linking agent BS^3. EGFR and the two mutants forms, kd and Wa5, underwent equivalent levels of homodimerization indicating that neither mutation affected dimerization (Fig. 6B). Dimers with the two mutants showed absence of tyrosine phosphorylation, suggesting that Wa5 may function as a dominant negative receptor.

We next investigated the effect of co-expressing mutant receptors with wild-type EGFR to determine the mechanism underlying the $Egfr^{Wa5}$ antimorphic allele. Wild-type *EGFR* expression vector (0.2 μg) was co-transfected individually or with half (0.1 μg) or equal (0.2 μg) molar ratios of wild-type *EGFR*, $EGFR^{kd}$ or $EGFR^{Wa5}$ expression vectors. The total amount of expression vector used was in the linear range of total EGFR expression (Fig. 6A). Total EGFR levels increased proportionally with increasing quantities of co-transfected vector for each of the three expression vectors (Fig. 6C). Similarly, EGFR phosphorylation levels increased proportionally with the quantity of wild-type *EGFR* expression vector when transfected alone. However, phosphorylation of total EGFR remained constant in cells co-transfected with the $EGFR^{kd}$ expression vector despite increasing levels of total EGFR, suggesting that the kd mutant, although kinase dead, does not inhibit the activity of wild-type *EGFR* when co-transfected with an equal molar ratio. Interestingly, phoshorylation of total EGFR decreased in proportion to the level of $EGFR^{Wa5}$ expression vector. Less than 10% of wild-type EGFR phosphorylation levels was detected in the cells co-transfected with equal molar ratios of the $EGFR^{Wa5}$ and wild-type *EGFR* expression vectors compared to wild-type *EGFR* alone. This result establishes that the Wa5 receptor can potently inhibit activation of wild-type EGFR. Furthermore, this result suggests that EGFR inhibition by the Wa5 mutant is due to a novel mechanism since the kd mutant, which also lacks a functional kinase, did not inhibit EGFR activation; and since classical cytoplasmic-tail truncated dominant negative receptors require over-expression to inhibit EGFR signaling (Xie et al. 1997).

To directly compare the phosphorylation status of EGFR-EGFR, EGFR-kd and EGFR-Wa5 complexes, we used the FLAG (f) epitope to tag the N-termini of the mature receptors, after the signal peptide. CHO cells were transiently co-transfected with wild-type *EGFR* expression vector and an equal quantity of wild-type $EGFR^{f}$, $EGFR^{fkd}$, or $EGFR^{fWa5}$ FLAG-tagged expression vectors. This permitted an analysis of the phosphorylation status of fEGFR, fkd, and fWa5 after immunoprecipitation of cell extracts using an anti-FLAG antibody (Fig. 6D). The three transfections had similar levels of EGFR and FLAG reactivity after immunoprecipitation with an anti-FLAG antibody indicating they had similar expression of FLAG-tagged protein in each transfection. However, the phosphorylation of fkd was dramatically reduced in cells co-transfected with *EGFR* and $EGFR^{fkd}$ expression vectors suggesting that two active kinases are required for optimal EGFR activation and that kd is phosphorylated, albeit at reduced levels, by EGFR. Nonetheless, dimerization of fkd and EGFR still resulted in phosphorylation indicating that EGFR-fkd homodimers have kinase activity. Conversely, phosphorylation was not detected in EGFR-fWa5 complexes indicating that Wa5 cannot be phosphorylated by EGFR and that EGFR-Wa5 homodimers lack kinase activity. EGFR expression vectors with C-terminal FLAG tags exhibit similar results to the N-terminal tagged proteins showing that the location of the FLAG tag does not adversely affect activation of EGFR (data not shown).

Our results using co-transfections with wild-type EGFR suggested that Wa5 inhibits EGFR activation by inducing or preventing a conformational change that inactivates the EGFR kinase domain or by preventing the receptor from becoming a substrate in a transphosphorylation reaction. To distinguish between these two potential mechanisms, we used the hyper-activatable Dsk5, produced by the $EGFR^{Dsk5}$ hypermorph allele (Fitch et al. 2003). Co-transfection of $EGFR^{Dsk5}$ and $EGFR^{Wa5}$ expression vectors revealed that Wa5 does not inhibit Dsk5 kinase activity (Fig. 6E); Wa5 can be a substrate for Dsk5. EGFR phophorylation increased proportionally to the quantity of $EGFR^{Wa5}$ expression vector co-transfected with $EGFR^{Dsk5}$ suggesting that Wa5 was phosphorylated by hyper-activatable

Dsk5. This result provides evidence that Wa5 inhibits wild-type EGFR by disrupting kinase activation within oligomeric complexes rather than by inducing a conformational change leading to defective substrate utilization.

EGFR^{Wa5} *does not inhibit EGFR-ERBB2 heterodimeric complexes*

Similar to Dsk5, the kinase of the EGFR-related protein, ERBB2, has much greater activity with constitutive activation when over-expressed. Since ERBB2 is the preferential heterodimerization partner for EGFR and other ERBB receptors (Graus-Porta et al. 1997), we determined whether Wa5 also inhibits heterodimeric complexes with ERBB2. *ERBB2* expression vector (0.1 μg) was co-transfected individually or with 0.1 μg, 0.3 μg or 0.5 μg of *EGFR*, *EGFR^{kd}* or *EGFR^{Wa5}* expression vectors. Total EGFR levels were proportionally increased with increasing quantities of co-transfected vector for all three *EGFR* expression vectors (Fig. 7A). However, ERBB2 levels decreased proportionally with increasing quantities of co-transfected *EGFR* expression vector. Co-transfection of *ERBB2^{K732M}*, coding for a kinase dead mutant of ERBB2, and *EGFR^{kd}* expression vectors exhibited similar results showing that the reduction in ERBB2 levels was independent of the phosphorylation status of either EGFR or ERBB2, and most likely is a result of transcriptional interference or altered intracellular trafficking as previously reported (Worthylake and Wiley 1997).

Phosphorylation of both EGFR and ERBB2 increased in cells co-transfected with wild-type *EGFR* and *ERBB2* expression vectors in proportion to the level of total EGFR (Fig. 7A). However, with *EGFR^{kd}* and *EGFR^{Wa5}* co-transfections, the level of ERBB2 phosphorylation was dependent on the level of ERBB2 and was not reduced by increasing levels of kd or Wa5. Interestingly, co-transfection of *ERBB2* and either *EGFR^{kd}* or *EGFR^{Wa5}* expression vectors resulted in phosphorylation of kd and Wa5, respectively (Fig. 7A), although kd and Wa5 alone did not exhibit phosphorylation (Fig. 6A). We also

immunoprecipitated the respective receptors for western blot analysis to confirm this result since separation of EGFR and ERBB2 by SDS-PAGE is often ambiguous in cells over-expressing both receptors. Equal quantities of *ERBB2* expression vector (0.1 μg) was co-transfected with 0.3 μg or 0.5 μg of wild-type *EGFR*, $EGFR^{kd}$, or $EGFR^{Wa5}$ expression vectors followed by immunoprecipitation of the cell extracts with either anti-EGFR or anti-ERBB2 antibodies (Fig. 7B). The immunoprecipitation-western blot analysis produced a similar result as shown in Fig. 7A; Wa5 phosphorylation levels were slightly reduced compared to that of kd when controlled for EGFR levels. However, both EGFR mutants exhibited phosphorylation indicating that ERBB2 phosphorylates these EGFR mutants and that ERBB2 kinase activity is not inhibited by either kd or Wa5.

Discussion

We determined that *Wa5*, an ENU-induced dominant mutation, is the result of a single base change in the coding region for a highly conserved DFG motif within the EGFR kinase domain. Mice carrying this mutation have wavy coats and partially formed eyelids at birth, similar to mice homozygous for the recessive $Egfr^{wa2}$ and $Tgfa^{wa1}$ mutations. Recently, we also reported that mice carrying the dominant $Egfr^{Dsk5}$ allele, also ENU-induced, have wavy coats and increased footpad pigmentation (Fitch et al. 2003). Dsk5 has elevated tyrosine kinase activity and is produced by a hypermorphic allele of *Egfr*. In contrast to the viability of $Egfr^{Dsk5}$ and $Egfr^{wa2}$ homozygous mice, homozygosity for $Egfr^{Wa5}$ is embryonic lethal. Failure of the $Egfr^{Wa5}$ allele to complement the $Egfr^{tm1Mag}$ null allele suggested that $Egfr^{Wa5}$ is functionally inactive.

$Egfr^{Wa5}$ *is an antimorphic allele*

Although the product of *Egfr*^{Wa5} appears to lack activity based upon an inability to complement the *Egfr*^{tm1Mag} null allele, several lines of evidence strongly suggest that *Egfr*^{Wa5} functions genetically as an antimorphic allele. By definition, an antimorphic allele heterozygous with a wild-type allele produces a more severe phenotype than a null allele heterozygous with a wild-type allele (Muller 1932). *Egfr*^{Wa5} meets this criterion since heterozygous *Egfr*^{Wa5}/+ mice have coat and eye defects while *Egfr*^{tm1Mag}/+ mice have no detectable abnormal phenotype.

Analysis of the developmental defects caused by Wa5, when compared with those observed in *Egfr*^{tm1Mag} embryos, supports the classification of *Egfr*^{Wa5} as an antimorphic allele. On most genetic backgrounds, homozygous *Egfr*^{tm1Mag} embryos die around 11.5 dpc, showing severe placental defects and a total loss of normal placental morphology by 13.5 dpc (Threadgill et al. 1995; Strunk et al. submitted). In placentae from *Egfr*$^{tm1Mag/tm1Mag}$ embryos at 10.5 dpc, development of multinucleated labyrinthine trophoblast cells is observed with disorganization of the labyrinthine trophoblast cell layer and formation of blood pockets (Threadgill et al. 1995). However, mice heterozygous for *Egfr*^{tm1Mag} have normal placental architecture. Similar to *Egfr*^{tm1Mag} homozygous embryos, the development of placentae from *Egfr*$^{Wa5/Wa5}$ embryos is impaired as early as 10.5 dpc, with a reduction in placental size and thickness identical to that observed in *Egfr*^{tm1Mag} homozygous null placentae. While *Egfr*$^{Wa5/Wa5}$ placentae have similar histological characteristics to placentae from *Egfr*^{tm1Mag} homozygous embryos such as the presence of the multinucleated labyrinthine trophoblast cells and the formation of blood pockets, superficially the *Egfr*$^{Wa5/Wa5}$ placentae appear to be less severely affected between 10.5-12.5 dpc compared to those from *Egfr*^{tm1Mag} homozygous mice on a 129 genetic background. Since the *Egfr*^{Wa5} allele is on a heterogeneous genetic background, it is probable that segregating modifier genes affect the ability of *Egfr*$^{Wa5/Wa5}$ embryos to survive until later in gestation. This is exactly the situation with *Egfr*^{tm1Mag} homozygous embryos on other inbred or mixed genetic backgrounds suggesting that *Egfr*^{Wa5} and *Egfr*^{tm1Mag} homozygous mice are morphologically

identical. However, unlike *Egfr*^{tm1Mag}, embryos heterozygous for *Egfr*^{Wa5} have minor morphological abnormalities in the spongiotrophoblast and labyrinthine trophoblast layers of the placenta although they are completely viable.

As noted above, mice lacking *Egfr* exhibit a strong genetic background-dependent variation in embryonic survival to term, and surviving EGFR-deficient pups show abnormalities in epithelial-derived tissues (Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995). Lung immaturity and post-natal brain degeneration are primarily responsible for the loss of surviving neonatal EGFR-deficient mice. Although a reduction in survival has not been observed for *Egfr*$^{wa2/wa2}$ mice on mixed genetic backgrounds, *Egfr*$^{wa2/Wa5}$ compound heterozygous mice show peri-natal lethality, severe growth retardation, complete hair loss, and craniofacial malformations, phenotypes also observed in *Egfr* null mice, indicating that the *Egfr*^{Wa5} allele exacerbates the reduced activity of the hypomorphic *Egfr*^{wa2} allele. *Egfr*$^{wa2/tm1Mag}$ compound heterozygous mice have no overt abnormalities other than a wavy coat on mixed genetic backgrounds (Roberts et al. 2001). Interestingly, we also showed a strong genetic interaction with the major EGFR ligand, TGFA, in *Tgfa*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ double mutants; these mutants exhibit progressively more severe post-natal growth retardation. Similar to *Egfr*^{wa2} homozygous mice, *Tgfa*$^{tm1Dcl/tm1Dcl}$ mice do not manifest aberrant growth (data not shown).

Analogous genetic interactions between *Egfr* and genes in the EGFR signaling pathway have been genetically revealed by generating *Egfr*$^{wa2/wa2}$ mice containing heterozygous mutations in genes coding for proteins downstream of the EGFR. For example, the incompletely penetrant eye defects seen in *Egfr*^{wa2} homozygous pups were enhanced by loss of one copy of *Sos1* (Wang et al. 1997); *Egfr*$^{wa2/wa2}$, *Sos1*^{tm1}/+ double mutant pups also showed elevated levels of peri-natal mortality. Likewise, heterozygosity for a null allele of *Shp2* dominantly enhanced peri-natal mortality, eyelid abnormalities, and epithelial defects (Qu et al. 1999), as well as semilunar heart valve enlargement in *Egfr*^{wa2} homozygous pups (Chen et al. 2000a). Since targeted mutations in the ligands for EGFR

show subtle to no abnormalities (Luetteke et al. 1999), the $Egfr^{Wa5}$ allele will be a useful tool to identify activities of individual EGFR ligands in the context of a mutant receptor.

Lastly, we used a functional biological assay to validate the antimorphic properties of $Egfr^{Wa5}$. We recently showed that Apc^{Min}-induced intestinal polyp number is dramatically reduced on a homozygous $Egfr^{wa2}$ background (Roberts et al. 2001). Although the $Egfr^{Wa5}$ allele reduced the number of Apc^{Min}-induced tumors, the reduction was not as dramatic as shown with $Egfr^{wa2/wa2}$ mice. This difference may be related to differences in residual kinase activity in $Egfr^{wa2/wa2}$ and $Egfr^{Wa5}$/+ mice, and potentially suggesting that the $Egfr^{Wa5}$ mutation is a more accurate model for pharmacological inhibition of EGFR since these mice still have one wild-type *Egfr* allele. Alternatively, these differences may be due to the heterogeneous background of $Egfr^{Wa5}$ mice used in this study; an extensive study into the effect of genetic background on placental defects in $Egfr^{tm1Mag/tm1Mag}$ mice indicates that EGFR-deficiency modifiers are numerous (Strunk et al. submitted). Similarly, multiple modifiers of Apc^{Min} and *Egfr* may be segregating differently. In spite of the genetic heterogeneity, an apparent synergy exists between *Egfr* and *Mom1* consistent with our previous observations (Roberts et al. 2001).

Wa5 has a dead kinase and inhibits wild-type EGFR

Since all *in vivo* evidence suggested that Wa5 has a functionally inactive kinase, potentially acting as a dominant negative receptor encoded by an antimorphic allele, we performed *in vitro* analysis to measure its intrinsic kinase activity. These results verified that Wa5, although capable of forming ligand-induced dimers, has a completely defective kinase. Furthermore, co-expression experiments demonstrated that Wa5 is a potent inhibitor of wild-type EGFR phosphorylation, an activity not observed with other EGFR kinase dead EGFR receptors.

An analysis of the crystal structure of the kinase catalytic domain, first determined for cyclic AMP-dependent protein kinase A (PKA) (Knighton et al. 1991), provides insight into the mechanism of Wa5 kinase inactivation. Using the PKA structure, many models for kinase activation have been developed and although a number of very different regulatory mechanisms exist for protein kinases, the catalytic domains of Ser/Thr and Tyr kinases adopt strikingly similar structures when they are in the "on" state (Johnson et al. 1996; Huse and Kuriyan 2002). The catalytic domain consists of two lobes; the N-terminal lobe is composed of five β sheet strands and one α helix (helix αC) while the C-terminal lobe is predominantly helical. ATP is bound in a deep cleft between the two lobes and proper ATP alignment is coordinated by a Gly-rich motif and the Lys^{72}-Glu^{91} ion pair (in PKA) within the phosphate-binding loop. Lys^{72} makes crucial contacts with the α and β phosphates of ATP and a mutation causing a change in the corresponding residue in EGFR, Lys^{721}, results in an impaired kinase (Chen et al. 1987), probably by dramatically reducing the binding affinity for the Mn·ATP complex (Cheng and Koland 1998). Emanating from the catalytic loop, the highly conserved Asp^{166} is oriented properly by hydrogen bond interaction with Asn^{171} and acts as a base for proton removal from a protein hydroxyl side chain of the substrate. A mutation causing a change in the corresponding Asp^{166} residue in EGFR (Asp^{813}) also abolishes kinase activity (Coker et al. 1994), probably due to an impaired phosphotransfer reaction although no change in nucleotide binding occurs (Cheng and Koland 1998).

Of the loops composing the catalytic domain, the conformation of the activation loop is the most markedly changed in the "on" state by phosphorylation to allow substrate binding to the activation loop. The conformation of the DFG motif, situated at the base of the activation loop and absolutely conserved in all active protein kinases (Hanks et al. 1988), is tightly coupled to phosphorylation of the activation loop. The Asp residue within the DFG motif is required for the binding of two divalent cations involved in ATP recognition.

The *Wa5* mutation, causing a change of the Asp residue, probably impairs not only ATP binding properties but also proper orientation for catalysis.

Changes in the DFG motif are found in the naturally existing dead kinases, CCK4/PTK7 and RYK (Kroiher et al. 2001). Interestingly, conversion of DNA to a DFG motif in RYK restores kinase activity even though RYK has changes in other conserved residues (Katso et al. 1999). Conversely, ERBB3, an impaired receptor tyrosine kinase of the EGFR/ERBB family, has a conserved DFG motif but has changes in other conserved residues (Guy et al. 1994).

Point mutations creating changes in the activation loop can also cause constitutive or heightened ligand-induced activation of receptor tyrosine kinases; for example, in proteins coded by the alleles $Egfr^{Dsk5}$, $Fgfr3^{K650E}$, Kit^{D816V} and Met^{D1246N} (Webster et al. 1996; Jeffers et al. 1997; Longley et al. 1999; Fitch et al. 2003). It is intriguing that a change in the fourth amino acid after the DFG motif occurs in the constitutively active kinases $EGFR^{Dsk5}$, KIT^{D816V} and MET^{D1246N}. An amino acid change of Val^{157} in avian c-erbB, corresponding Val^{702} in EGFR, that is located close to the Gly-rich sequence motif shows increased or decreased phosphorylation depending upon the substituted amino acids (Shu et al. 1990). These results indicate that the tertiary structure of the kinase domain and the correct relative orientation appear crucial for catalysis and improper interactions of the kinase domain with Mn·ATP and substrates cause either diminution or constitutive activation of catalytic activity.

The structure-function analysis of various mutant receptors with inactive kinases suggests that Wa5 has both abnormal ATP binding and utilization, a situation significantly different than the kinase dead kd mutant; kd mutant receptors have weak ATP binding but when bound, ATP can be utilized. This dichotomy appears at odds with current dimer models of EGFR activation; activated dimers alone cannot adequately explain the dominant negative activity of Wa5 and are thus not compatible with the antimorphic activity of $Egfr^{Wa5}$.

Tetramer model of EGFR activation

Since the *in vivo* and *in vitro* data strongly suggests that a simple dimer model cannot explain the potent antimorphic activity of $Egfr^{Wa5}$, we propose that an alternative tetrameric or oligomeric model is more consistent with the activity of Wa5. Structural analysis of protein kinases in "on" and "off" states has revealed that conformational plasticity is central to the mechanism of kinase activity regulation. Autophosphorylation within the activation loop of the kinase domain causes conformational changes, removing substrate binding inhibition and properly positioning the catalytic groups (Huse and Kuriyan 2002). However, the tyrosine kinase domain of EGFR is highly unusual in that phosphorylation of the activation loop does not occur to promote its activity (Gotoh et al. 1992). The crystal structure of the unphosphorylated EGFR kinase domain suggests that the EGFR activation loop adopts a conformation observed only in other kinases that are phosphorylated and activated (Stamos et al. 2002).

Although it is well established that ligand-induced dimerization increases the tyrosine kinase activity of EGFR, the mechanism by which EGFR kinase is activated is not clearly understood (Schlessinger 2000). It is thought that ligand-stabilized extracellular dimerization induces cytoplasmic dimerization in which the activation loops are stabilized in a conformation favorable for catalysis. Our study suggests that EGFR-Wa5 dimers do not support a conformation necessary for catalysis and thus Wa5 acts as dominant negative like EGFR truncation mutants lacking most of the cytoplasmic domain (Kashles et al. 1991). A simple dimmer model would suggest that $EGFR^{Wa5}$/+ cells should have at least 25% of wild-type activity, much greater than what we actually observed. Rather, similar to the dominant-negative effect of the KIT^{E839K} mutant on wild-type KIT phosphorylation (Longley et al. 1999), a more drastic reduction of phosphorylation in CHO cells suggests that EGFR-Wa5 dimers may interfere with kinase activity of wild-type dimers through

tetramer or high-order inter-molecular process between ligand-stabilized dimers (Sherrill 1997)(Fig. 8A).

Consequently, a model that is more consistent with our analysis of Wa5 consists of membrane-bound pre-dimers, stabilized by ligand binding, that are not yet fully activated. Rather than the 25% of normal kinase activity level predicted in *EGFR*^{Wa5}/+ cells based upon a dimer model, we observed substantially less, lower than 10% activity suggesting that the signal has been further diluted. More consistent with this observation is tertameric or high-order complexes underlying the activated receptors (Honegger et al. 1989; Murali et al. 1996; Huang et al. 1998). Using a tetramer model, only one-sixteenth of the complexes would be fully active; all others would contain at least one Wa5 receptor, thus disrupting full activation of the receptor complexes. Therefore, our model suggests that dimers become stabilized upon ligand binding, but must aggregate to become fully activated. Upon activation, they could then efficiently propagate a signaling cascade. This model raises the important question, can dimers associate and dissociate to activate other dimers. We predict that there is a quarternary complex that is required to activate members within the respective dimers of the tetrameric complex and that these complexes are essentially kinase dead when containing a Wa5 receptor and incapable of further aggregation to activate EGFR-Wa5 dimers. An intriguing possibility is that upon stabilization of dimers by ligand binding, the dimers are internalized and it is within cytoplasmic vesicles that these higher order complexes form (Wang et al. 2002; Wiley 2003). Removal from the cell surface provides one potential mechanism by which Wa5 can permanently inhibit complexes containing wide-type receptors and preventing disassociation and reassociation of wild-type EGFR dimers. Although we cannot formally rule out that Wa5 forms stable, inactive dimers with EGFR (Fig. 8A), our data suggests otherwise. We would have observed a greater level of interaction between EGFR and Wa5 in the FLAG-tagged experiments if this were the case.

The model we propose for homodimeric interactions is also consistent with heterodimeric interactions (Fig. 8B). Previous studies have suggested that ERBB2 remains

in cytoplasmic vesicles in non-transformed cells while EGFR is predominately on the cell surface (Worthylake and Wiley 1997), consistent with the lack a known ligand for ERBB2. Upon EGFR activation, ERBB2 becomes trans-phosphorylated, most likely by internalized vesicles containing ligand-stabilized EGFR dimers. This model also suggests that EGFR and ERBB2 do not form stabilized dimers but rather are part of tetrameric or higher-order complexes. Furthermore, this model is conistent with lateral signal propagation between activated receptors (Graus-Porta et al. 1997). Lastly, phosphorylation of Wa5 by ERBB2 suggests that Wa5 does not inhibit these higher-order heteromeric complexes when highly active kinases are present. Thus, this is also consistent with the inability of Wa5 to inhibit Dsk5 mutant receptors that act very similar to ERBB2.

The crystal structure of the extracellular domain of EGFR has provided significant insights into how ligand induced dimerization occurs but our genetic and molecular studies of *Egfr*^{Wa5} suggest that questions still remain to explain the specificity of homodimerization, heterodimerization and higher-order receptor oligomerization (Burgess et al. 2003). Although we cannot dismiss a raft model of activation whereby activated complexes are dependent upon local signal density that is disrupted in the presence of Wa5 receptors, we favor the tetramer model described above. The analysis of *Egfr*^{Wa5} has provided new clues into the complexity of EGFR/ERBB signaling and with additional structural analysis of activated signal complexes, will help resolve many unanswered questions in receptor-mediated signal propagation.

Materials and methods

Egfr^{Wa5} *mutation identification*

All exons (Reiter et al. 2001), along with immediate flanking regions, of *Egfr* were amplified by PCR from *Egfr*^{Wa5}/+, BALB/c, C3H and C57BL/6 genomic DNA using intronic primers

that were also used for subsequent sequence analysis. PCR products were purified using the Multi-screen PCR 96-well filtration system (Millipore) on a Biomek 2000 robotic platform (Beckman) and sequenced directly using Big Dye™ terminator cycle sequencing (ABI). Sequences were analysed using the Sequencer™ program to identify the causative mutation in *Egfr*^{Wa5}.

Mouse strains crosses and genotyping

The *Egfr*^{Wa5} mutation was maintained by intercrossing *Egfr*^{Wa5}/+ mice on a mixed genetic background containing contributions from BALB/c, C3H and C57BL/6J. The *Egfr*^{wa2}/+ and *Egfr*^{tm1Mag}/+ mutations were maintained on C57BL/6J congenic or 129S6/SvEvTAC isogenic backgrounds, respectively. *Tgfa*^{tm1Dcl} null mice were maintained on a mixed genetic background of 129S6/SvEvTAC and C57BL/6J (Luetteke et al. 1993). The *Apc*^{Min} mutation was maintained as congenic on a C57BL/6J background.

Complementation studies were performed by crossing *Egfr*^{tm1Mag}/+ females with *Egfr*^{Wa5}/+ males. *Egfr*^{Wa5}/+ female mice were bred to *Apc*^{Min}/+ male mice, producing *Apc*^{Min}/+ mice on wild-type and *Egfr*^{Wa5}/+ backgrounds. To generate *Tgfa*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ double mutant mice, *Tgfa*$^{tm1Dcl/tm1Dcl}$ females were mated to *Tgfa*^{tm1Dcl}/+, *Egfr*^{Wa5}/+ males. In order to generate compound heterozygous animals, *Egfr*^{wa2}/+ females were crossed to *Egfr*^{Wa5}/+ males. The morning a vaginal plug was detected was defined as 0.5 day of embryonic development (0.5 dpc). Mice were fed Purina Mills Lab Diet 5058 under specific pathogen free conditions in an American Association for the Accreditation of Lab Animal Care approved facility. Mice were euthanized by CO_2 asphyxiation.

Embryonic yolk sacs from embryos or tail clips from neonates were used to extract DNA for genotype determination by PCR. The genotype for the *Egfr*^{Wa5} allele was determined by PCR using three primers: mEgfr-Ex21-S5, 5'-gcatgtcaagatcacaga-3'; mEgfr-Ex21-S6, 5'-gcatgtcaagatcacagg-3'; and mEgfr-In21-As1, 5'-tagagaatgaccctgacgag-3'. The

mEgfr-Ex21-S5 and mEgfr-In21-As1 primers amplify a 228 base pair (bp) product specific for the wild-type *Egfr* allele and primers mEgfr-Ex21-S6 and mEgfr-In21-As1 amplify a 228 bp product specific for the $Egfr^{Wa5}$ allele. Genotypes for the $Egfr^{tm1Mag}$, $Egfr^{wa2}$, Apc^{Min}, *Mom1* and $Tgfa^{tm1Dcl}$ alleles were determined by PCR as previously described (Luetteke et al. 1993; Roberts et al. 2001).

In vivo *phosphorylation assays*

Neonatal pups were injected subcutaneously with 10 μl/g body weight of phosphate-buffered saline (PBS) or 0.5, 1.0 or 10 μg/g body weight of EGF (R&D Systems) in PBS. After ten minutes, liver and skin were harvested, frozen in liquid nitrogen and stored at –80°C. The frozen tissues were homogenized in 5 to 10 volumes (5 to 10 ml/g tissue) of homogenization buffer consisting of 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, 1 mM sodium vanadate and 10 mM β-glycerophophate at 4°C. The tissue lysates were cleared by centrifugation for 10 min at 4°C and protein concentrations were determined by the Bradford assay (Bio-Rad). An equal amount of protein lysate (15 μg) was separated by denaturing 7.5% SDS-PAGE, and transferred to PVDF membranes (Bio-Rad). Protein blots were incubated with recombinant anti-phosphotyrosine antibody conjugated with horseradish peroxidase (Pharmingen) or polyclonal rabbit anti-EGFR antibody (Neomarker), and detected with an enhanced chemiluminesence system (Amersham-Pharmacia).

Cell culture and transfection

Chinese hamster ovary (CHO) cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, and

penicillin/streptomycin. CHO cells in 60-mm dishes at 50-70% confluency were transfected with human *EGFR* or *ERBB2* expression vectors in pcDNA3.1 (Invitrogen) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) and cell extracts were prepared at 24 hours after transfection as described previously (Fitch et al. 2003). The Asp855Gly (Wa5) and Lys745Met (kd) changes were introduced into a human *EGFR* expression vector by site-directed mutagenesis using GeneEditor (Promega) and the entire coding regions verified by sequence analysis. An equal amount of protein (12 μg) was separated by denaturing 6% SDS-PAGE and analyzed by western blot analysis as described above.

Chemical cross-linking

Chemical cross-linking of EGFR was carried out as described previously (Qian et al. 1994). Briefly, CHO cells transfected with EGFR expression vectors in 6-well plates for 24 hours were washed twice with cold PBS. One milliliter of PBS containing 2 mM bis(sulfosuccinimidyl) suberate (BS^3) (Pierce) was added and incubated at room temperature for 30 min with rocking of the plates. The reaction was stopped by washing twice with cold 20 mM Tris-HCl, pH 7.4, 150 mM NaCl and cell lysates were prepared as described above. Protein concentration was measured using the Bradford assay (BioRad). An equal amount (12 μg) of protein was separated by 6% SDS-PAGE, transferred to PVDF membranes and subjected to western blot analysis as described above.

FLAG-tag and immunopreciptation-western blot

A *Xba* I to *Dra* III fragment of the human EGFR expression vector was replaced with a *Sac* I to *Dra* III fragment of pBK-Flag-EGFR (Kim et al. 2003) containing the FLAG epitope immediately after the signal sequence to generate N-terminal FLAG-tagged wild-type (fWt), kd (fkd) and Wa5 (fWa5) EGFR expression vectors. Wild-type *EGFR* expression vectors

were co-transfected with an equal quantity (0.2 μg) of *EGFR*^{f}, *EGFR*^{fkd} or *EGFR*^{fWa5} expression vector and cell extracts were prepared 24 hours after transfection as described above. Cell lysates (200 μg) were immunoprecipitated with 5 μg of monoclonal anti-FLAG M2 antibody (Sigma) overnight at 4°C and then for 2 hours after addition of 30 μl of Protein G-agarose (Pierce). Immunoprecipitated complexes were washed three times with TBS buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and the bound proteins were eluted by boiling for 5 min in 2x SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue). For the immunoprecipitation of EGFR or ERBB2, cell lysates (100 μg) were incubated overnight with 5 μg of monoclonal anti-EGFR antibody (clone 528) or 3 μg of polyclonal anti-ERBB2 antibody (Santa Cruz) at 4°C. After 2 hours of incubation with 30 μl of Protein G-agarose, immunoprecipitated complexes were washed four times with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and the bound proteins were eluted as described above. One-sixth of the immunoprecipitate volumes were separated by 6% SDS-PAGE, transferred to PVDF membranes and subjected to western blot analysis as described above. For the detection of the FLAG epitope, the same antibody was used for western blot analysis as for immunoprecipitation.

Intestinal macroadenoma analysis

The small intestines from pylorus to caecum and the colons were dissected from three-month-old mice, flushed gently with PBS to remove fecal material, cut longitudinally and splayed flat on Whatmann 3MM paper. The gastrointestinal tracts were fixed overnight at 4°C in 10% neutral buffered formalin (Sigma) before storing in 70% ethanol. The number and diameter of macroadenoma polyps were characterized as previously described (Roberts et al. 2001). The Mann-Whitney paired rank sum test was used to analyze the association

between genotype and polyp number and size. Statistical analysis was performed using StatView (SAS Institute).

Placental histology

Placentae were collected at 12.5 dpc from embryos produced by intercrossing $Egfr^{Wa5}$/+ on a mixed genetic background, washed in PBS and fixed in 10% neutral buffered formalin overnight. The fixed placentae were washed in PBS and 0.9% saline, dehydrated in a series of ethanols and xylenes and embedded in paraffin. After cutting and mounting seven-micron sections on slides, they were deparaffinized, rehydrated in a graded series of ethanols and counterstained with hematoxylin and eosin. Stained sections were dehydrated in a series of ethanols and coverslips mounted with permount. Representative histological images were photographed at a magnification of 200X using a CCD digital camera.

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Table 1. Wa5 *and* Egfr^{tm1Mag} *complementation cross*

Genotype[a]				Litters
+/+	*Wa5/+*	*Egfr^{tm1Mag}/+*	*Egfr^{tm1Mag}/Wa5*	
7	14	6	0	3

[a] Number of viable 3-day old neonates

Table 2. *Viability of* Egfr^{Wa5} *homozygotes from* Egfr^{Wa5}/+ *intercrosses*

Stage[a]	Genotypes[b]			Litters
	+/+	*Egfr*^{Wa5}/+	*Egfr*$^{Wa5/Wa5}$	
10.5 dpc	12(0)	26(2)	14(4)	8
12.5 dpc	22(0)	40(0)	6(3)	10
14.5 dpc	13(0)	30(1)	6(2)	8
P0	20	40	3	11
P21	20	39	0	11

[a] dpc, embryonic day and P, postnatal day

[b] Number of normal (abnormal) embryos or live pups

Table 3. *Viability of* Egfr$^{wa2/Wa5}$ *compound heterozygotes*

Stage[a]	Genotype[b]				Litters
	+/+	*Egfr*^{wa2}/+	*Egfr*^{Wa5}/+	*Egfr*$^{wa2/Wa5}$	
P0	35	19	32	16	14
P21	34(1)	17(2)	31(1)	4(12)	12

[a] P, postnatal day

[b] Number of live (dead) pups

Figure legends

Figure 1. Sequence and structure of Wa5 mutation. (*A*) Top, DNA and amino acid sequence comparison of *Egfr*^{Wa5} (*Wa5*) kinase region containing the ENU-induced mutation with wild-type *Egfr* (+). Bottom, amino acid sequence similarity between highly divergent human Ser/Thr and Tyr kinases. All DFG-containing kinases have a functional kinase while those lacking the DFG motif, below the dotted line, are kinase dead. (*B*) Ribbon structure of human EGFR kinase domain (PDB, 1M14) displayed with Rasmol showing positions of amino acids altered in different *Egfr* mutations. Lys^{723} and Asp^{815} have been proven to be essential for kinase activity *in vitro*. Sites of existing *Egfr* mutations in mice (and their alleles) are indicated. The amino acid numbering is based upon mature mouse EGFR sequence.

Figure 2. Histological analysis of placentae from 12.5 dpc embryos. (A) Placenta from wild-type *Egfr* embryo stained with hematoxylin and eosin. Formation of decidua (De), trophoblast giant cell (arrowheads), spongiotrophoblast (Sp) and labyrinthine (Lt) layers was normal. (*B*) Placenta from *Egfr*^{Wa5}/+ embryo with reduced Sp and formation of multinucleated trophoblast cells (arrows). (*C*) Placenta from phenotypically normal *Egfr*$^{Wa5/Wa5}$ embryo with reduced Sp layer. (*D*) Placenta from severely growth retarded *Egfr*$^{Wa5/Wa5}$ embryo. The embryo attached to this placenta was 70% smaller than wild-type littermates. Formation of multinucleated trophoblast cells, a highly disorganized Lt layer and blood pockets were observed. Scale bar = 50 μm.

Figure 3. Phenotypes of *Egfr*$^{wa2/Wa5}$ compound and *Tgfa*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ double mutant pups. (*A*) Representative *Egfr*$^{wa2/Wa5}$ compound mutant (front) with hair loss and eye defects compared to *Egfr* wild-type littermate (back). (*B*) Narrow, elongated snout, underdeveloped low jaw and loss of whiskers were also observed in *Egfr*$^{wa2/Wa5}$ compound

mutants. (*C*) Two *Tgfa*$^{tm1Dcl/tm1Dcl}$, *Egfr*^{Wa5}/+ double mutant pups (right) with representative growth retardation compared to four *Egfr* wild-type littermates (left). The mice in *A* and *B* were two-month-old and in *C*, 2-week-old.

Figure 4. Effect of *Egfr*^{Wa5} on *Apc*^{Min}-mediated intestinal tumorigenesis. (*A*) Polyp number in *Apc*^{Min}/+ mice with and without an *Egfr*^{Wa5} allele. Each dot represents the total number of polyps (>0.3 mm in diameter) from individual three-month-old mice. Horizontal lines are means for each genotype. n=20 for *Egfr* wild-type and n=22 for *Egfr*^{Wa5}/+. (*B*) Sizes of polyps (diameter) from tumor-bearing mice in *A*. Each dot represents the mean size of all polyps from a single mouse. Horizontal lines are means for each group.

Figure 5. EGF-induced tyrosine phosphorylation *in vivo*. (*A*) Liver and skin extracts from two-day-old pups. (*B*) Liver extract from seven-day-old pups. Wild-type *Egfr* (+/+) and *Egfr*^{Wa5}/+ (Wa5/+) pups were injected subcutaneously with either PBS (P) or EGF ($E_{0.5}$: 0.5, $E_{1.0}$: 1.0 and E_{10}: 10 μg/g of body weight) before euthanizing 10 minutes later. Extracts were prepared and equal amounts (15 μg) were analyzed by western blot analysis using either anti-phophotyrosine antibody (pY) or anti-EGFR antibody (EGFR). kD, molecular weight markers; arrowheads, EGFR; arrows, p120 and p55 phosphorylation targets.

Figure 6. Western blot analysis of extracts from CHO cells transiently transfected with wild-type and mutant *EGFR* expression vectors. (*A*) Levels of total EGFR (EGFR) and phosphorylated EGFR (pY) protein in extracts from wild-type *EGFR* (Wt), *EGFR*^{kd} (kd) or *EGFR*^{Wa5} transfected CHO cells. Cells were transfected with 0.1, 0.2 or 0.5 μg of the respective expression vector. (*B*) Dimerization complexes of Wt, kd and Wa5 in extracts from CHO cells transiently transfected with 0.5 μg of respective expression vector followed by chemical cross-linking. Arrowheads, monomers; arrows, dimers. (*C*) Levels of total and phosphorylated EGFR in extracts from CHO cells transiently co-transfected with 0.2 μg of

wild-type *EGFR* expression vector and 0.1 or 0.2 μg of Wt, kd or Wa5 *EGFR* expression vectors. (*D*) Immunoprecipitation-western blot analysis of extracts from CHO cells transiently co-transfected with wild-type *EGFR* expression vector and FLAG-tagged (f) Wt, kd or Wa5 *EGFR* expression vectors. After immunoprecipitation with anti-FLAG antibody, immune complexes were divided equally before electrophoresis and blotting to three different membranes for detection of EGFR, FLAG and pY. (*E*) Levels of total and phosphorylated EGFR in extracts from CHO cells transiently co-transfected with 0.25 μg of $EGFR^{Dsk5}$ expression vector and 0.05, 0.1 or 0.25 μg of $EGFR^{Wa5}$ expression vector or vice versa. C, extracts from non-transfected CHO cells; EGFR, anti-EGFR antibody; pY, anti-phosphotyrosine antibody; FLAG, anti-FLAG antibody; triangles, dilution series of co-transfected constructs.

Figure 7. Western blot analysis of extracts from CHO cells transiently transfected with *ERBB2* and mutant *EGFR* expression vectors. (*A*) Levels of total EGFR (EGFR), total ERBB2 (B2) and phosphorylated EGFR or ERBB2 (pY) in extracts from cells co-transfected with 0.1 μg *ERBB2* (B2) expression vector alone or with 0.1, 0.3 or 0.5 μg of wild-type *EGFR* (Wt), $EGFR^{kd}$ (kd) or $EGFR^{Wa5}$ (Wa5) expression vectors. (*B*) Levels of immunoprecipitated (IP) EGFR, ERBB2 and phosphorylated EGFR or ERBB2 in extracts from cells co-transfected with *ERBB2* expression vector and wild-type *EGFR*, $EGFR^{kd}$ or $EGFR^{Wa5}$ expression vectors as described for *A*. Only 0.3 and 0.5 μg of *EGFR* expression vectors are shown. After immunoprecipitation with anti-EGFR or anti-ERBB2 antibodies, the immune complexes were divided equally into three aliquots before electrophoresis and blotting to three different membranes for detection of EGFR, ERBB2, and pY. Triangles, dilution series of co-transfected constructs.

Figure 8. Model for Wa5 antimorphic action. *(A)* EGFR monomers form ligand-independent pre-dimers and, in one model, irreversible EGFR-Wa5 homodimers are

preferentially formed. Upon ligand binding, dimers become stabilized and internalized, although they do not posses a fully activated kinase. When EGFR-Wa5 or Wa5-Wa5 homodimers form, they remain as inactive kinases. However, inter-dimer interactions may play major role in fully activating the kinases of wild-type receptor. Only one out of 16 combinations in this model will have EGFR dimer-dimer interactions, thus resulting in a dramatic reduction in overall ligand-induced phosphorylation when an equal numbers of EGFR and Wa5 receptors exist. *(B)* Unlike EGFR,which is predominately on the cell surface, ERBB2 is primarily in cytoplasmic vesicles. Similar to the homodimers, ligand-stabilized EGFR dimers get internalized and can then form hetero-tetrameric interactions with ERBB2. Since the kinase of ERBB2 has much greater activity than EGFR, complexes with Wa5 do not inhibit phosphorylation. Blue, black and orange lines indicate EGFR, Wa5 and ERBB2 receptors, respectively. Single lines, monomers ; double lines, dimers; green circles, ligand; black ovals, inactive tyrosine kinases; orange explosions, active kinases; P, phosphorylation sites.